Reduced-Size Antagonists of Luteinizing Hormone-Releasing Hormone Active in Vitro†

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A series of reduced-size analogs of LHRH was designed with the length varying from nine to two amino acids. These compounds were tested in vitro for the LH suppression in cultured rat pituitary cells treated with 1 ng of LHRH. The best analogs were also tested in vivo for their antiovulatory activity in rats. It appeared that terminal amino acids as well as the presence of Arg or ILys in the sequence are both crucial for the antagonism. The most potent antagonist in this series was a heptapeptide, AcDNal-Ser-Tyr-DNal-Leu-Arg-ProNHEt, which completely inhibited LH release at the dose 0.1 μg and inhibited ovulation at 1000 μg/rat. For fragments shorter than heptapeptide the inhibition of LH release was observed at the dose 100 μg of the analog.

Introduction

The luteinizing hormone-releasing hormone (LHRH, pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH$_2$) plays a major role in the modulation of reproductive functions. Inhibitors of LHRH have been logical candidates for new types of antifertility agents. However, the relatively low potency and high cost of the competitive antagonists, coupled with the finding that some of them release histamine, have been the main obstacles to their acceptance and use.

Over the last 10 years a few thousand LHRH antagonists were synthesized and tested.[1,2] The majority of them were decapeptides which contained from four to six unnatural amino acids, many of them in the D-configuration. To date not much has been published on analogs of LHRH having less than 10 amino acids. Haviv et al.[3] reported a series of hexapeptide analogs of LHRH, both agonists and antagonists, which contained only one D-amino acid in their sequence and were designed on the 3–9 fragment of the agonist leuprolide, which is [DLeu^6,Pro^{9}NHEt]LHRH. The best compound was [*N*-[3-(1-naphthyl)propionyl]Ser^4,DNal^6,Pro^{9}NHEt]-LHRH$_{4-9}$. It displayed very potent receptor binding affinity and a high antagonist potency in releasing LH from cultured rat pituitary cells. Another series of reduced-size analogs also reported by Haviv et al.[4] was based on the 2–9 fragment of LHRH. The best analog in this series was [*N*-[(4-fluorophenyl)propionyl]-D-1-Nal^3,NMeTyr^5,DNicLys^6,ILys^8,DAla^{10}]LHRH$_{3-10}$ which was equipotent with Nal-Glu^5 in suppressing LH in the castrated rat model.

Herein, we report a new series of reduced-size LHRH analogs. Their length varied from nine to two amino acids. They were tested in vitro for LH inhibition in cultured rat pituitary cells and some of them in vivo for their antiovulatory activity in rats.

† Abbreviations of the unnatural amino acids: Nal, 3-(2-naphthyl)-alanine; Cpa, 3-(4-chlorophenyl)alanine; Pal, 3-(3-pyridyl)alanine; ILys, N^ϵ-isopropyllysine; PicLys N^ϵ-(2-pyridylcarbonyl)lysine (N^ϵ-picolinoyllysine).

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Chemical Synthesis

All the peptides were synthesized by solid phase method (SPPS). Peptides containing Gly-NH$_2$, DAla-NH$_2$, or Pro-NH$_2$ at the C-terminus were synthesized on benzhydrylamine (BHA) resin, and were cleaved with anhydrous HF.[6] Peptides containing ProNHEt at the C-terminus were synthesized using Boc-Pro attached to Merrifield resin and were cleaved from the resin with ethylamine,[7] followed by removing all protective groups with anhydrous HF. The purified peptides were characterized by analytical HPLC, FAB-MS, and AAA.

Biological Testing

Antagonists were tested in vitro for LH suppression in cultured rat pituitary cells treated with 1 ng of LHRH,[8] and some of them in vivo for their antiovulatory activity in rats.[9]

Results and Discussion

The goal of this research was to find the shortest fragment of the LHRH antagonist that would significantly inhibit LH release in vitro. Thirty-two analogs recorded in Table 1 were all assayed at several of the following dosages: 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 μg to determine LH antagonist activity.

The decapeptide **1**, [AcDNal^{1,6},DCpa^2,DPal^3,DAla^{10}]-LHRH, was chosen as a parent compound. **1** inhibited completely the release of LH at the dose 0.1 μg. Introduction of pGlu instead of DNal was detrimental for the antagonist activity (analog **2**). Nonapeptide without DNal (analog **3**) was also inactive. Elimination of the amino acid in position 2 or 3 or both 2 and 3 (peptides **4**–**6**) produced antagonists which inhibited LH release at 1 μg/mL. Further modifications of octapeptide **6** involved changes in positions 5 and/or 6 (**7**–**9**), replacement of Arg by ILys (**10**), and replacement of DAla at the C-terminus by Gly (**11**). None of these changes was successful. Further shortening of the peptide chain by replacing the C-terminal Pro-DAlaNH$_2$ by ProNHEt resulted in heptapeptide **12** which was equipotent to parent decapeptide **1** and was the most potent reduced-size antagonist of this series. The congeners of this antagonist with changes in positions 6 and 8 and at the C-terminus (**13**–**19**) were all less

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Table 1. In Vitro Antagonist Activity of LHRH Analogs

		control		analog (mg) + LHRH (1 ng)							
no.	sequence	–	LHRH (1 ng)	0.03	0.1	0.3	1.0	3.0	10	30	100
1	AcDNal-DCpa-DPal-Ser-Tyr-DNal-Leu-Arg-Pro-DAlaNH$_2$	3.8 ± 0.8	149 ± 6	8 ± 2	4 ± 0	4 ± 1	4 ± 1				
2	pGlu-DCpa-DPal-Ser-Tyr-DNal-Leu-Arg-Pro-DAlaNH$_2$	3.8 ± 0.8	132 ± 3	165 ± 2	158 ± 0	170 ± 5	149 ± 1				
3	AcDCpa-DPal-Ser-Tyr-DNal-Leu-Arg-Pro-DAlaNH$_2$	3.5 ± 0.3	151 ± 8	152 ± 6	165 ± 6	152 ± 5	145 ± 1	77 ± 2			
4	AcDNal-DPal-Ser-Tyr-DNal-Leu-Arg-Pro-DAlaNH$_2$	3.8 ± 0.8	149 ± 6	28 ± 2	14 ± 0	6 ± 1	3 ± 1				
5	AcDNal-DCpa-Ser-Tyr-DNal-Leu-Arg-Pro-DAlaNH$_2$	3.8 ± 0.8	149 ± 6	39 ± 2	29 ± 2	14 ± 3	6 ± 1				
6	AcDNal-Ser-Tyr-DNal-Leu-Arg-Pro-DAlaNH$_2$	5.3 ± 1	236 ± 15		37 ± 1	12 ± 0	7 ± 2	3 ± 1			
7	AcDNal-Ser-Tyr-DPal-Leu-Arg-Pro-DAlaNH$_2$	4.8 ± 1	251 ± 29		112 ± 5	72 ± 6	33 ± 0	11 ± 0			
8	AcDNal-Ser-Tyr-DPicLys-Leu-Arg-Pro-DAlaNH$_2$	6.3 ± 1	241 ± 29		201 ± 18	154 ± 8	67 ± 3	29 ± 0			
9	AcDNal-Ser-PicLys-DPicLys-Leu-Arg-Pro-DAlaNH$_2$	7.2 ± 0.8	224 ± 22		118 ± 13	60 ± 1	26 ± 7	8 ± 1			
10	AcDNal-Ser-Tyr-DNal-Leu-ILys-Pro-DAlaNH$_2$	2.1 ± 0.2	149 ± 1	127 ± 4	135 ± 2	104 ± 9	55 ± 2	23 ± 2			
11	AcDNal-Ser-Tyr-DNal-Leu-Arg-Pro-GlyNH$_2$	3.8 ± 0.8	132 ± 5		157 ± 2	112 ± 0	50 ± 1	45 ± 1			
12	AcDNal-Ser-Tyr-DNal-Leu-Arg-ProNHEt	3.2 ± 1	153 ± 15	45 ± 1	5 ± 0	4 ± 1	3 ± 0				
13	AcDNal-Ser-Tyr-DPal-Leu-Arg-ProNHEt	9 ± 2	279 ± 17		134 ± 7	69 ± 2	34 ± 5	14 ± 2			
14	AcDNal-Ser-PicLys-DPicLys-Leu-Arg-ProNHEt	2.1 ± 0.8	149 ± 5	108 ± 3	72 ± 2	27 ± 4	8 ± 0				
15	AcDNal-Ser-Tyr-DTrp-Leu-Arg-ProNHEt	2.1 ± 0.8	149 ± 5	85 ± 6	40 ± 2	10 ± 1	2 ± 0				
16	AcDNal-Ser-Tyr-DNal-Leu-ILys-ProNHEt	3.5 ± 0.2	148 ± 1	146 ± 4	133 ± 2	26 ± 9	6 ± 2	4 ± 2			
17	AcDNal-Ser-Tyr-DPicLys-Leu-ILys-ProNHEt	3.5 ± 0.3	151 ± 8	194 ± 6	200 ± 2	157 ± 2	118 ± 10				
18	AcDNal-Ser-Tyr-DNal-Leu-Arg-ProNH$_2$	4.1 ± 2	167 ± 8	154 ± 4	74 ± 2	13 ± 1	4 ± 2	3 ± 1			
19	AcDNal-Ser-DNal-Leu-Arg-Pro-DAlaNH$_2$	2.3 ± 0.1	155 ± 4		117 ± 1	37 ± 0	13 ± 0	4 ± 1			
20	AcDNal-Ser-Tyr-DNal-Leu-ProNHEt	3.1 ± 1	145 ± 0			148 ± 5	155 ± 1	151 ± 3	55 ± 2	11 ± 6	
21	AcDNal-Tyr-DNal-Arg-DAlaNH$_2$	2.3 ± 0.1	156 ± 13				142 ± 6	135 ± 1	121 ± 2	66 ± 9	21 ± 3
22	AcDNal-DNal-Leu-Arg-ProNHEt	2.3 ± 0.1	156 ± 13				145 ± 16	158 ± 1	147 ± 2	122 ± 9	98 ± 4
23	AcDNal-PicLys-DPicLys-Arg-DAlaNH$_2$	2.3 ± 0.1	156 ± 13				145 ± 16	158 ± 1	142 ± 2	105 ± 9	64 ± 2
24	AcDNal-Leu-Arg-DAlaNH$_2$	3.5 ± 0.4	155 ± 4				145 ± 4	136 ± 17	116 ± 9	87 ± 4	49 ± 3
25	AcDNal-PicLys-Arg-DAlaNH$_2$	2.3 ± 0.1	156 ± 4				147 ± 3	145 ± 1	75 ± 4	26 ± 11	8 ± 2
26	AcDNal-DPicLys-Arg-ProNH$_2$	3.5 ± 0.4	153 ± 13				148 ± 11	145 ± 2	132 ± 2	136 ± 1	63 ± 2
27	AcDNal-DPicLys-ILys-ProNH$_2$	3.5 ± 0.4	153 ± 13				186 ± 4	150 ± 2	144 ± 10	128 ± 5	118 ± 7
28	AcDNal-Leu-Arg-ProNHEt	3.1 ± 0	145 ± 2				148 ± 11	120 ± 7	88 ± 5	25 ± 4	9 ± 2
29	AcDNal-Arg-DAlaNH$_2$	3.5 ± 0.4	153 ± 13				151 ± 3	145 ± 3	101 ± 7	66 ± 3	69 ± 2
30	AcDNal-DPicLys-ProNH$_2$	3.1 ± 0	145 ± 2				145 ± 3	139 ± 4	129 ± 7	113 ± 5	71 ± 3
31	AcDNal-DAlaNH$_2$	3.5 ± 0.4	153 ± 13				148 ± 10	153 ± 7	124 ± 6	88 ± 3	16 ± 4
32	AcDNal-ProNHEt	3.1 ± 0	145 ± 2				135 ± 9	141 ± 6	101 ± 7	59 ± 6	14 ± 1

potent. Hexapeptide **20** that was void of Arg was completely inactive even at high doses. All shorter fragments were tested starting from the dose of **1** μg. Tetrapeptides **25** and **28** showed strong inhibition at 100 μg. Surprisingly, also, two dipeptides tested (**31** and **32**) showed inhibition at this dose level.

The best analogs were also tested in vivo for their antiovulatory activity (AOA) in rats. The doses of

Table 2. In Vivo Antiovulatory Activity of LHRH Analogs

	AOA (rats ovulat/total rats) dose, μg			
analog no.	1	10	100	1000
1	5/6	0/6		
4		6/6	6/6	4/6
5			6/6	6/6
6			6/6	6/6
12			6/6	2/9

antagonists injected were 1, 10, 100, or 1000 μg/rat. The decapeptide **1** inhibited completely ovulation at 10 μg/rat. The best reduced-size analog **12** inhibited ovulation at 1000 μg/rat. The in vivo data are shown in Table 2.

Conclusions

Several conclusions have emerged from these studies that might be important for further development of reduced-size and non-peptide LHRH analogs.

The first observation is that acetylated DNal, which was the most commonly used substituant for position 1 in decapeptide LHRH antagonists seems to be very important for the antagonism also in the reduced-size analogs. $DAlaNH_2$ at the C-terminus can be in some sequences replaced by ProNHEt, but $ProNH_2$ and $GlyNH_2$ are usually less effective. As it was a case with the decapeptide LHRH antagonists, the presence of Arg or ILys is necessary, but Arg is usually a better choice. Positions 2 and 3 of the LHRH sequence are not essential for the antagonism and can be omitted. The middle part of the molecule is most flexible to changes and will be the main variable of our future designs.

Experimental Section

Instruments. All the peptides were synthesized using Beckman 990 peptide synthesizer. The HF-reaction apparatus, Type 1B, was from Peninsula Laboratories. Analytical reversed-phase HPLC was run on a Waters instrument with 660 solvent programmer and a Vydac C_{18} column, 25 cm × 4.6 mm i.d. Amino acid analyses were carried out on a Beckman 118-CL amino acid analyzer after hydrolysis in constant boiling 6 N HCl for 24 h, using standard procedures. The unnatural amino acids were qualitatively determined with the exception of Pal which was quantified. FAB-MS was run using a 5995 Hawlett-Packard instrument.

Starting Materials. The following protected amino acids Boc-Ser(OBzl), Boc-Tyr(O-2Br-Cbz), Boc-Leu, Boc-Arg(Tos), Boc-Pro, Boc-Gly, Boc-DAla, and Boc-DTrp as well as BHA resin and Boc-Pro Merrifield resin were purchased from Advanced ChemTech. Boc-DNal, Boc-DCpa, Boc-DPal, and Boc-ILys(Cbz) were synthesized at the Southwest Foundation for Biomedical Research and made available by the Contraceptive Development Branch, Center for Population Research, NICHD. Boc-L- and -D-PicLys was prepared in our laboratory.[10] All the solvents were purchased from Fisher Scientific, and all other chemicals were obtained from Aldrich.

General Synthetic Method for the SPPS and Purification of Peptides 1–32. A typical synthesis of a peptide used 0.4 g of BHA resin for peptides containing $GlyNH_2$, $DAlaNH_2$, or $ProNH_2$ at the C-terminus or 0.4 g of Boc-Pro-Merrifield resin for peptides with ProNHEt ending. The synthetic protocol and the conditions for cleavage of the resin and protecting groups for peptides containing $GlyNH_2$, $DAlaNH_2$, or $ProNH_2$ were identical as described earlier.[11] For peptides containing ProNHEt, ethylamine was used as a cleaving reagent,[7] followed by HF deprotection. Purification of all the peptides was achieved by gel filtration on Sephadex G-25 with 6% acetic acid as the eluant, followed by chromatography on Sephadex LH-20. The solvent system was water:1-butanol: acetic acid:methanol, 90:10:10:8. The purity of the peptides was checked by analytical HPLC using a C_{18} peptide column (25 cm × 4.6 mm). The solvent system was 0.1% TFA in water/acetonitrile, and the gradient was 55–80% acetonitrile over 30 min. The flow rate was 1.5 mL/min, and the absorbance was measured at 254 nm. The purity of the peptides was estimated to be over 95%.

In Vitro Biological Assay. The in vitro test was performed as described: Anterior pituitaries of female Sprague–Dawley rats weighing 240–260 g (18 rats) were removed after decapitation. Tissue was minced and placed in Gey's solution with (1) trypsin, (2) DN-ase I, and (3) LBBI. Cells were washed and centrifuged between each addition. After dispersion, cells were washed with DMEM containing 0.45% glucose and 25 mM HEPES along with other reagents (2 mM glutamine, 0.1 mM pyruvate, 2% antimycine, 0.3% fungizone, 0.1% NEAA, 2.5% FCS, 3% ES, 10% fresh RS, 1 nM T, 100 nM dexamethasone) and plated. Each 1 mL of DMEM contained approximately 3.0×10^5 cells. After a 4-day incubation at 37 °C with 8% CO_2, cells were washed and fresh Gey's solution with 0.3% glucose was added to each well. Peptide antagonists and LHRH were added to the wells, and the stimulation time was 2 h. Media was removed and assayed for LH by RIA. The RIA reagents were obtained from NIADDK (reference), Dr. G. Niswender (antibody), and Dr. L. Reichert (iodinating hormone). LH levels were recorded in terms of ng/mL of the LH reference RP-3.

The AOA was determined in rats as described[9] by counting on estrus the number of ova by 4-day cycling rats, after a single subcutaneous injection of the LHRH analog in corn oil, which was administered between 12 and 12:30 p.m. on proestrus.

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Supporting Information Available: Amino acid analysis data and FAB-MS spectrometry data (4 pages). Ordering information is given on any current masthead page.

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